EVALUATING THE BEHAVIORAL AND PHYSIOLOGICAL SAFETY OF HUMAN BUTYRYLCHOLINESTERASE IN RHESUS MONKEYS

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ABSTRACT

We evaluated a large dose of human butyrylcholinesterase (Hu-BChE) in rhesus monkeys using a complex cognitive test (serial-probe recognition, SPR) designed to assess attention and short-term memory. Concurrent with the cognitive-behavioral assessment, blood was collected at critical points throughout the study and was analyzed for AChE and BChE activity, anti-BChE (antibody) activity, and gross clinical pathology (i.e., complete blood count and CHEM 20 panel). Each monkey received an injection of 150 mg (105,000 U or 32 mg/kg) of Hu-BChE 60 min prior to testing on the SPR task. No cognitive-behavioral decrements of any kind were detected in SPR performance and no robust or consistent signs of clinical pathology were detected in any of the blood assays during the 5 weeks of observation. The pharmacokinetics of intravenous Hu-BChE administration revealed a peak in BChE of 226 U/ml at 5 minutes post injection and a mean residence time of approximately 72 hours. These results provide strong support for the behavioral and physiological safety of Hu-BChE in rhesus monkeys.

INTRODUCTION

Classic nerve agents, such as sarin, soman and VX, are extremely toxic organophosphate (OP) compounds that pose a serious threat to both civilian and military sectors. These agents are believed to produce their lethal effects by irreversibly binding to the enzyme acetylcholinesterase (AChE), leading to acetylcholine (ACh) accumulation at synaptic sites and hyperactivity of the cholinergic system (Fonnum & Guttormsen, 1969; Shih, 1982). If left untreated, nerve agent poisoning results in a rapid progression of symptoms, including convulsions, hypersecretion, and death due to cardiovascular and respiratory failure (Dunn & Sidell, 1989; Shih, 1982). The current treatment for OP intoxication consists of a combination of compounds including carbamates, antimuscarinics, an oxime nucleophile (reactivator) and anticonvulsants under pre- and post-exposure conditions (Doctor, Raveh, Wolfe, Maxwell, & Ashani, 1991; Lenz, Broomfield, Maxwell, & Cerasoli, 2001; Wilson & Ginsburg, 1955). Unfortunately, this therapy for nerve agent exposure fails to provide complete protection, and concomitant side effects often result from some of the drugs employed (Castro, Larsen, Finger, Solana, & McMaster, 1992; Leadbeater, Inns, & Rylands, 1985; McDonough, Jaax, Crowley, Mays, & Modrow, 1989; McDonough & Shih, 1997).

To prevent OP injury and lethality and to eliminate adverse behavioral side effects, one potential approach is to use enzymes such as cholinesterases as *pretreatment* drugs (Allon et al., 1998; Ashani et al., 1991; Broomfield et al., 1991; Doctor, Maxwell, Ashani, Saxena, & Gordon, 2001; Doctor et al., 1991; Maxwell et al., 1992; Raveh et al., 1989; Raveh, Grauer, Grunwald, Cohen, & Ashani, 1997; Wolfe et al., 1992; Wolfe, Rush, Doctor, Koplovitz, & Jones, 1987). A clear advantage of using this strategy over the current multiple-drug regimen is that this single agent may provide more than adequate protection against OP intoxication and death. Moreover, early evidence suggests that protection may be afforded in the absence of behavioral side effects (for review see Doctor et al., 2001; Lenz et al., 2001). For example, Genovese and Doctor (1995) injected rats with butyrylcholinesterase derived from equine serum (Eq-BChE) and tested them in a battery of behavioral tasks including passive avoidance, locomotor activity, and a variable interval schedule of reinforcement. No significant alterations in behavior were observed in these tasks following exposure to Eq-BChE. Using rhesus monkeys (*Macaca mulatta*), Matzke and colleagues (1999) and Broomfield et al. (1991) failed to observe any decrements in cognitive-behavioral performance on a six-item serial probe recognition (SPR) task, a test of attention and short-

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term memory, following exposure to Eq-BChE. Performance on a primate equilibrium platform was similarly unimpaired in rhesus monkeys following administration of Eq-BChE, despite cumulative soman challenges equivalent to three times the LD50 (Wolfe et al., 1992).

While the behavioral safety of Eq-BChE is well established, effects of BChE derived from *human* plasma (Hu-BChE) have been investigated in only a limited way. Hu-BChE protected rats against soman exposure and performance in the Morris water maze was unaffected 1 week after treatment (Brandeis, Raveh, Grunwald, Cohen, & Ashani, 1993). To be employed effectively, Hu-BChE must provide protection against nerve agent without producing undesirable behavioral effects when administered alone. To this end, we examined the neurobehavioral effects of Hu-BChE on trial completion, choice accuracy, and choice latency in a six-item SPR task in rhesus macaques. This procedure has been demonstrated to be a sensitive and reliable paradigm for evaluating disruptions in cognitive-behavioral functioning (attention and memory) following challenges by drugs affecting the cholinergic system (Castro, 1995, 1997; Myers et al., 2002). Additionally, considerable behavioral data from humans and rhesus monkeys performing SPR tasks allows for more direct comparison between these two species (Sands & Wright, 1980a, 1980b; Wickelgren & Norman, 1966; Wright, Santiago, Sands, Kendrick, & Cook, 1985). Indeed, the SPR task originated as a paradigm for studying memory functioning in humans (Wickelgren and Norman, 1966; Ellis and Hope, 1968; Wickelgren, 1970). To more fully and comprehensively characterize the safety of Hu-BChE in rhesus monkeys, we analyzed blood samples throughout the study to determine cholinesterase activity and pharmacokinetics, antibody production, and clinical blood pathology resulting from intravenous Hu-BChE administration. We used a large dose of Hu-BChE to increase the likelihood of detecting any toxic effects.

METHOD

The experimental protocol was approved by the Animal Care and Use Committee at the Walter Reed Army Institute of Research and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

1. SUBJECTS

Four adult female rhesus monkeys (*Macaca mulatta*) weighing between 4.3 – 4.9 kg served. All monkeys were experimentally naïve with respect to the behavioral procedures and apparatus at the start of the study. The monkeys were housed individually in stainless steel squeeze-back cages (61 cm W X 71 cm D X 86 cm H) with free access to tap water. Certified primate rations (Purina Mills, Inc., St. Louis, MO) were provided no sooner than 30 min after the daily session to maintain desired body weights at approximately 90% of free-feeding weight. The colony was maintained on a 12-h light/dark cycle with no twilight (lights on at 0600 h) and at 20-22°C with a relative humidity of 50% (±15%), using at least 10 complete air changes per hour of 100% conditioned fresh air. The sessions began at approximately 1100 and were conducted during the light part of the cycle, 5 days per week (Monday – Friday).

2. COGNITIVE-BEHAVIORAL APPARATUS

The subjects were tested unrestrained in their home cages. A 35.6-cm (14-in.) capacitive touch screen monitor (GoldStar StudioWorks, model GLD 45I, Microtouch Systems, Inc., Methuen, MA) was attached to the front wall of each cage, with the center of the screen 38.9 cm above the chamber floor. Because screen touches are difficult to execute around the screen's perimeter, the effective area of the screen was reduced by 1.5 cm on all four sides. Banana-flavored food pellets (750 mg, Bio-Serv Inc., Frenchtown, NJ) were delivered by a pellet dispenser (BRS/LVE Model QNB-400 1) into a food cup (7.9 X 10.8 X 7.6 cm) positioned in the front of the test chamber, accessible through an aperture (7.6 cm wide X 5.4 cm high) centered 15.1 cm below the lower edge of the touch screen and 11.6 cm above the chamber floor. A computer, running a custom-written Visual Basic® 6.0 routine, was used to control experimental events and collect all data.

3. SERIAL-PROBE RECOGNITION PROCEDURE

Each daily session consisted of 240 trials. On each trial, 6 unique sample stimuli (list items) were presented sequentially, separated by a 0.5-s interstimulus interval (ISI) during which the screen was blank. Each list item was a compound stimulus comprised of two superimposed, randomly selected

ASCII characters of different size and color. The individual characters ranged from about 0.3 to 2.7 cm in length and 0.3 to 2.7 cm in width. Because the same ASCII character could be selected for a particular sample stimulus, one character was 15% smaller than the other and was offset slightly above and to the left of the other to avoid perfect overlap and to achieve a greater diversity of compound sample stimuli. The RGB color saturation of each ASCII character ranged from 0 to 255. To exclude extremely dark characters but not true colors, at least one of the three saturation levels had to exceed 79. Each list stimulus was displayed in the top-center portion of the screen, about 13.5 cm from the left edge of the screen and about 4 cm from the top of the screen to the center of the stimulus. Each list item was presented for 4 s or until it was touched (with the restriction that a minimum of 0.5 s had elapsed), at which point it was terminated and the ISI was initiated. After presentation of the sixth sample stimulus, the screen was blank throughout the 1-s probe delay (retention interval) that preceded the choice period. During the 15-s choice period a probe stimulus was displayed in the lower-left or lower-right portion of the screen, and a standard or default stimulus (a 6.6-cm white square) was presented in the other portion of the screen, with equal frequencies of presentation on both sides. The probe item was a compound stimulus that matched a list item on half of all trials (120). Across these "matching" trials, probe items matched list items at each of the six serial positions with equal frequency (20 at each serial position). On matching trials, touching the probe stimulus was considered correct. In contrast, on "non-matching" trials the probe stimulus was not among those listed (novel) and touching the default stimulus was considered correct. A correct choice response immediately produced the conditioned reinforcer (a white screen for .25 s) each time and a food pellet 50% of the time, determined randomly by the computer. Touching the opposite stimulus was considered incorrect. Choice periods that elapsed without a response ended after 15 s and were considered incorrect. A 5-s intertrial interval (or ITI, during which the screen was blank) separated each trial, regardless of whether a choice was correct or incorrect. A response during the ITI reset the interval, although few such responses occurred.

4. PHARMACOLOGICAL PROCEDURES

Monkeys were trained to sit passively in a collar-restraint chair (Primate Products, Inc., Woodside, CA) and present either leg for blood collection/injection procedures. All blood samples were taken from the saphenous vein. Hu-BChE (150 mg) was dissolved into approximately 5.5 ml of room-temperature physiological saline. SPR testing began exactly 60 min after enzyme administration was completed. The enzyme injection was delivered into one saphenous vein and all blood samples were collected from the other leg to avoid any possible overestimation of circulating Hu-BChE. The 0-min (pre-injection baseline) blood sample was collected 1 week prior to enzyme administration. Post-injection blood samples were taken at 5 and 45 min, at 3.5, 7, 24, 48, and 72 hours, and at 1, 2, 3, and 4 weeks following enzyme injection. For all monkeys, a vehicle injection (5.5 ml saline) was given 5 days prior to enzyme administration and on Day 30 post injection. A few days after the second vehicle control, we injected physostigmine hemisulfate (i.e., Eserine; Sigma Chemical Co.) intramuscularly at a dose of 0.07 mg/kg 15 min before SPR testing as a positive control.

5. BLOOD ASSAY PROCEDURES

All blood samples were analyzed for AChE and BChE activity using 1 mM acetylthiocholine and 1 mM butyrylthiocholine as the substrates, respectively (Ellman et al., 1961). The presence of anti-Hu BChE antibodies was followed by ELISA, using 0.2 unit of Hu-BChE per well as the plate-coating antigen and 1:20 dilution of blood (Rosenberg et al., 2002). Monkey antibody binding to Hu-BChE was detected with peroxidase-labeled rabbit antibody to monkey IgG using ABTS substrate. Standard curves using purified monkey IgG were run with each assay to allow quantification of antibody response.

Hematology studies (complete blood count, CBC) were performed using an ABX Pentra 60 (ABX Diagnostics, Irving CA). All specimens were tested unaltered using flow cytometry technology. Chemistry studies (Chemistry Profile, CHEM20) were performed using a Vitros 250 (Ortho Clinical Diagnostics, a subsidiary of Johnson and Johnson). Serum collected from each centrifugated specimen was analyzed using colorimetric and point-rate technology. Blood samples subjected to the chemistry profile (CHEM 20) and complete-blood-count (CBC) were analyzed by three experts (an experienced clinical pathologist, a medical doctor, and a primate veterinarian) who searched for any signs of possible serum pathology.
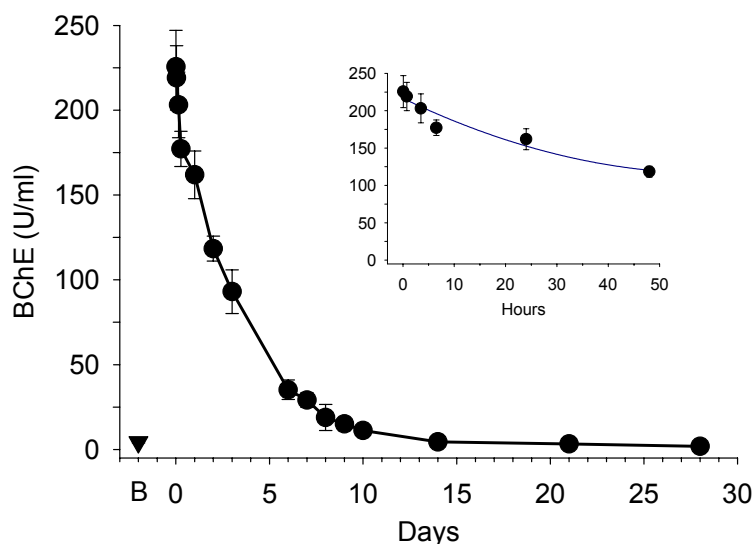

Figure 1. Circulating levels of BChE (U/mL) are shown for each day blood was taken. Baseline (B) represents BChE activity 1 week prior to enzyme administration. The first several post injection blood samples (5, 45, 210, and The inlay shows the same data in hours, across only the first 2 days post injection. Mean values are shown and error bars represent the standard error of the mean.

RESULTS

The time-course of circulating BChE levels is shown in Figure 1. The 0-min baseline (B) mean equaled 4.3 U/mL. At 5-min post injection, the mean level of BChE in the blood equaled 225.6 U/mL. After this peak at 5 min, BChE levels fell sharply. The mean residence time was 72 h. By 2 weeks post injection, the circulating level of BChE (4.4 U/mL) approximated baseline. The inlayed graph in Figure 1 shows the BChE level across the first 48 h of and the unit of measurement on the x-axis is hours rather than days.

Figure 2 summarizes critical aspects of SPR performance for all days of the experiment. The three measures of performance shown are the number of trials completed (top panel), accuracy (center panel), and choice response latency (bottom panel). Values represent the mean of each daily session for all four monkeys. Along the x-axis, the first vehicle injection is indicated by a V and administration of the Hu-BChE enzyme is indicated by an E. Each numeral represents the number of days following the injection of Hu-BChE. The rightmost column within each panel represents performance on the day physostigmine was injected at a dose of 0.07 mg/kg. The mean number of trials completed was consistently high and near the maximum of 240 throughout the experiment, but equaled only 88.5 on the day of physostigmine administration. Accuracy was approximately .836 for all sessions except the physostigmine positive control, which equaled .278. Mean choice response latencies were consistent for each subject, but differed considerably between subjects. Therefore, latency data were analyzed and plotted as a proportion of the mean latency of each subject on the first vehicle day.
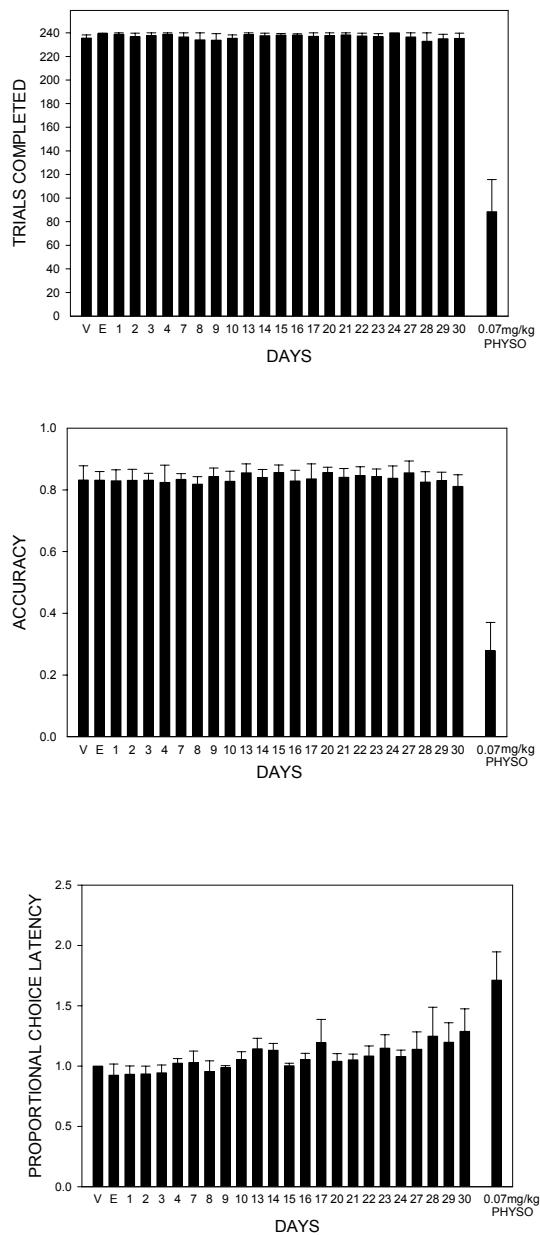
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Figure 2. Trials completed, accuracy, and proportional choice response latency are shown for each daily serial-probe recognition session. Vehicle (V), Enzyme (E) and days following enzyme administration are shown in addition to the physostigmine positive control (far right, 0.07 mg/kg PHYSO). Mean values are shown and error bars represent the standard error of the mean. Only trials in which a choice response was made were included in calculations of accuracy and reaction time. Trials completed, accuracy, and reaction time differed significantly from Vehicle only in the physostigmine session.

Proportional latencies were near 1.0 for all sessions except on the physostigmine injection day on which it equaled 1.71 (a relative increase of 71% from vehicle). Although a slight increase in mean proportional latency was observed across the last few sessions of the experiment, this increase was not statistically significant and resulted largely from increased response latencies for one monkey. No statistically significant differences were found for any of the three performance measures between either vehicle injection and enzyme administration or between the first and second vehicle injections. In contrast,

performance following physostigmine administration differed significantly from the first vehicle administration for all three measures and differed significantly from the second vehicle administration for number of trials completed and accuracy, but not latency.

Results from the serum and blood chemistry analyses revealed no signs of clinical pathology. For the most part, values were within the expected and normal ranges for adult rhesus monkeys. For a few measures (e.g., potassium, calcium, albumin, and MCHC), values were just above the normal range but did not represent a clinically significant departure from normal. Another measure, LYM#, was below the normal range throughout the experiment, both before and after injection of Hu-BChE. Thus, no signs of pathology were detected in blood or serum chemistry and no robust or consistent changes resulted from enzyme injection.

Figure 3 shows antibody levels in the blood across the experiment. IgG concentration peaked on Day 14 at a value of approximately 80 U/mL. Changes in antibody production were not correlated with any changes in behavior.
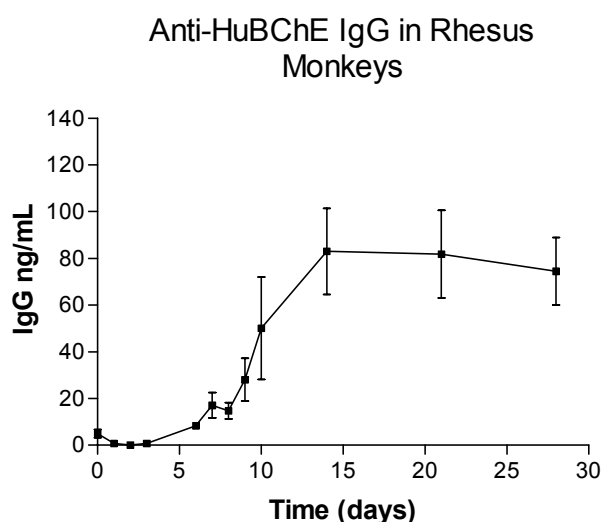

Figure 4. Anti-Hu-BChE antibody response (in ng/mL IgG) is shown for the entire study beginning with the day of enzyme administration. Mean values are shown and error bars represent the standard error of the mean.

CONCLUSIONS

A 150-mg (105,000 U or 32 mg/kg) intravenous injection of Hu-BChE produced no untoward cognitive-behavioral effects on motivation, accuracy, or reaction time in rhesus monkeys performing the SPR task. Circulating BChE levels peaked at 226 U/ml at 5 minutes post injection, the injected BChE had a mean residence time of approximately 72 hours, and circulating levels of BChE returned to pre-injection levels within 3 weeks. Antibody production peaked on day 14 at a modest level of 80 ng/mL IgG. Blood chemistry analyses revealed no indications of clinical pathology. No clinical signs of toxicity were observed at any time during the experiment. As a positive control, physostigmine hemisulfate (0.07 mg/kg) was administered and demonstrated that the SPR performance of these four monkeys was significantly disrupted on all three measures by cholinergic challenge. The data from this study support the following summary statements:

No disruption in performance on the SPR was observed following intravenous injection of 150 mg Hu-BChE. In contrast, physostigmine produced a substantial deficit on the task. Daily clinical observations revealed no signs of an adverse reaction. Therefore, a large dose of Hu-BChE appears behaviorally and physiologically safe in rhesus monkeys.

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